mTOR regulates expression of slit diaphragm proteins and cytoskeleton structure in podocytes

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Vollenbrüker B, George B, Wolfgart M, Saleem MA, Pavenstädt H, Weide T. mTOR regulates expression of slit diaphragm proteins and cytoskeleton structure in podocytes. Am J Physiol Renal Physiol 296: F418–F426, 2009. First published November 19, 2008; doi:10.1152/ajprenal.90319.2008.—The immunosuppressive mammalian target of rapamycin (mTOR) inhibitors can cause proteinuria, especially in kidney and heart transplanted patients. Podocytes play a major role in establishing the selective permeability of the blood-urine filtration barrier. Damage of these cells leads to proteinuria, a hallmark of most glomerular diseases. Interestingly, podocyte damage and focal segmental glomerulosclerosis can occur after treatment with an mTOR inhibitor in some transplant patients. To investigate the mechanisms of mTOR inhibitor-induced podocyte damage, we analyzed the effect of rapamycin on mTOR signaling and cellular function in human podocytes. We found that prolonged rapamycin treatment reduced the expression of total mTOR, which correlates with diminished levels of mTOR phosphorylation at Ser2448 and Ser2481. In addition, treatment with rapamycin reduced rictor expression and mTORC2 formation, resulting in a reduced phosphorylation of protein kinase B at Ser473. The expression level of the slit-diaphragm proteins nephrin and transient receptor potential cation channel 6 as well as the cytoskeletal adaptor protein Nck significantly decreased. Moreover, rapamycin reduced cell adhesion and cell motility, which was accompanied by an enhanced formation of dot-like actin-rich structures. Our data provide new molecular insights explaining which pathways and molecules are affected in podocytes by an imbalanced mTOR function because of rapamycin treatment.

sirolimus; proteinuria; slit diaphragm; mammalian target of rapamycin

RAPAMYCIN IS A POTENT IMMUNOSUPPRESSIVE drug with growth-inhibitory and anti-tumor properties. It inhibits mammalian target of rapamycin (mTOR), a large protein that is highly conserved during evolution. The serine/threonine kinase mTOR is part of two complexes, mTORC1 and mTORC2. The mTORC1 complex includes at least three additional components, a large associated protein called raptor, mLST8, and FKBP12 (6, 27, 43). The raptor-mTOR interaction seems to be crucial for TORC1 activity, since the formation of the rapamycin-FKBP12 complex interferes with the binding of raptor to mTOR, which results in an inhibition of TOR complex 1 (TORC1)-dependent phosphorylation of downstream targets that regulate translational efficiency (23, 43).

More recent studies identified a second complex, mTOR complex 2 (called TORC2). This complex includes mTOR, mLST8, Sin1, and rictor, a regulatory component that replaces raptor. The mTOR-rictor interaction cannot directly be influenced by rapamycin treatment, since the rictor-mTOR complex is not able to bind the FKBP12-rapamycin conjugate, suggesting that TORC2 in contrast to TORC1 is rapamycin insensitive (32). However, it was recently shown that prolonged rapamycin treatment influences TORC2 assembly (33). There is also increasing evidence that TORC2 plays a role in cytoskeletal organization (3, 32, 34, 44).

In contrast to calcineurin inhibitors (CNI), rapamycin has not been suggested to induce direct nephrotoxicity. Therefore, this drug is supposed to be an excellent candidate to replace CNI in therapy of chronic allograft nephropathy (8, 20, 29). However, a number of studies report that, in spite of the expected nonnephrotoxic effects, the conversion from CNI to rapamycin in patients after kidney or heart transplantation can induce proteinuria and occasionally nephrotic syndrome (1, 9, 18, 41). Rapamycin-associated proteinuria is mainly observed in patients who suffer from predamaged grafts, caused by already preexisting proteinuria, preexisting allograft glomerulopathy, or the recurrence of primary diseases such as focal segmental glomerulosclerosis (FSGS) (5, 8, 9, 18, 38, 41). Moreover, Letavernier et al. (24) reported podocyte damage and de novo induction of FSGS by rapamycin (24).

Podocytes are important for maintaining the selective filtration barrier of the renal glomerulus. They are complex cells that develop primary and secondary foot processes that form the slit diaphragm, an unique cell-cell contact that serves as a final filtration barrier (21, 30). The slit diaphragm is formed by specialized adhesion proteins that connect the foot processes, forming an open space between them. Nephrin, Neph family members, P-cadherin, and FAT are thought to form the extracellular parts of the slit diaphragm; intracellular components of the slit diaphragm are cytoskeletal adaptors like CD2AP, zonula occludens 1 (ZO-1), podocin, and α-actinin 4 (21, 30). Injury of the slit diaphragm or the actin cytoskeleton as well as interference with the podocyte-glomerular basement membrane (GBM) interaction have been shown to cause foot process effacement and proteinuria (21).

The aim of this study was to show how rapamycin affects mTOR signaling in podocytes and to elucidate possible mechanisms of rapamycin-induced podocyte damage. Here, we show that rapamycin affects the expression level of several components of the mTOR/protein kinase B (Akt) pathway as well as components of the slit diaphragm. Moreover, rapamycin...
cin treatment impairs the migration and adhesion of podocytes and leads to the induction of actin-rich dot-like structures.

METHODS

Cell culture and rapamycin treatment. Human immortalized podocytes (AB 8 cells) were cultivated as previously described (31). In brief, cells were grown in standard RPMI 1640 medium containing 10% FCS and supplements at the permissive temperature of 33°C (in 5% CO2) to promote cell propagation to 50–80% confluence (31). After that, cells were shifted to the nonpermissive temperature of 37°C (in 5% CO2) to allow terminal differentiation for 10 up to 14 days. Next, cells were treated with 10 ng/ml (Rapa 10) or 100 ng/ml (Rapa 100) rapamycin for 24 or 120 h, respectively. As a negative control, we used the solvent controls Control 10 (0.01% ethanol) or Control 100 (0.001% ethanol). Rapamycin (Sigma) was dissolved in ethanol at a concentration of 1 mg/ml (stock solution) and further diluted in distilled water.

Cell number and necrosis (lactate dehydrogenase release assay). The amount of necrotic cells from differentiated AB 8 cells cultured in 24-well plates was measured determining lactate dehydrogenase (LDH) activity. LDH is a stable cytosolic enzyme that is released in the medium upon cell lysis. LDH activity was assessed with a CytoTox 96w Non-Radioactive Cytotoxicity assay (Promega) following the manufacturer’s instructions. The amount of LDH released from necrotic cells was expressed as a percentage of total LDH, the sum of LDH released from the vital cells lysed by freeze/thaw cycles, and LDH in the medium (%cytotoxicity).

Apoptosis assay (annexin V assay). After experimental treatment, differentiated AB 8 cells were scraped from disposable six-well culture plates, collected in PBS, washed with fluorescence-activated cell sortor (FACS) buffer [PBS with Ca2+ and Mg2+, 0.5% FCS, 0.5% NaN3 (Merck)], and stained with annexin V fluorescein isothiocyanate (BD Biosciences, Pharmingen) 1:25 for 30 min at 4°C in the dark. Hereafter, cells were washed one time with FACS buffer and resuspended, and 104 cells were acquired using a FACScan Flow Cytometer (Becton-Dickinson). The analysis was performed using Cell Quest Software 3.3 (Becton-Dickinson). Apoptotic cells were expressed as a percentage of total cells.

Extract preparation and Western blot. Cellular lysates were prepared by scraping cells in immunoprecipitation (IP) buffer (1% Triton X-100, 20 mM Tris·HCl (pH 7.5), 25 mM NaCl, 50 mM NaF, 15 mM Na2PO4, and 1.5 mM EDTA) containing protease inhibitor (Complete; Roche). Lysates were centrifuged at 10,000 g for 30 min at 4°C. Supernatant (500 μl) was incubated with 5 μl of mTOR antibody (Cell Signaling Technology) for 90 min, rotated at 4°C to allow complex formation. Afterward, 25 μl of a 50% slurry of protein G-Sepharose were added, and incubation continued for 1 h. Unbound proteins were removed by washing the beads eight times with lysis buffer, and immunoprecipitates were analyzed by immunoblotting as described before.

Rho/Rac/Cdc42 activation assay. Cellular lysates from immortalized podocytes preincubated with 100 ng/ml rapamycin or solvent control for 24 h were prepared in 1× Mg2+ Lysis/Wash Buffer (Millipore). Total protein concentration ranged above 3.5 mg/ml. For the Rac and Cdc42 Activation Assay, lysates were preincubated with GST-Septosome for 45 min (GE Healthcare). The pulldowns were carried out following the manufacturer’s instructions. In brief, Rho/Rac/Cdc42 assay reagents specifically bind to GTP forms but not to GDP forms of the respective proteins. Precipitated activated Rho was detected with Anti-Rho (against RhoA, RhoB, and RhoC; 3 μg/ml); anti-Rac and anti-Cdc42 were diluted to 1 μg/ml (Millipore).

Immunofluorescence analysis. For indirect immunofluorescence analysis of cultured podocytes, cells were grown on collagen-coated cover slips and fixed in 4% paraformaldehyde supplemented with 4% sucrose in PBS at RT for 20 min. Samples were washed with PBS and then incubated for 10 min in 50 mM NH4Cl in PBS to quench reactive amino groups. After being washed with PBS, cells were permeabilized in PBS containing 0.2% Triton X-100 for 5 min and washed (3 times) in PBS containing 0.2% Triton X-100 and 0.2% gelatine (PBS-TG). Next, samples were blocked with 10% goat serum diluted in PBS-TG for 20 min at RT. Immunofluorescence staining was performed by incubating the cover slips for 1 h at RT with primary antibodies diluted in PBS-TG containing 2% goat serum. The antibodies against vinculin, zyxin, and cortactin were used in a 1:100 dilution. Cover slips were washed after antibody incubation in PBS-TG and incubated for 20 min at RT with fluorochrome-conjugated secondary antibodies (Molecular Probes) diluted in PBS-TG containing 2% goat serum and costained with Alexa Flour 488-phalloidin from Molecular Probes (1:40). After being washed in PBS, cover slips were rinsed in water, and cells were mounted in Crystal Clear Mount Medium (Sigma). Samples were examined with a conventional light microscope (Zeiss Axiosvert).

Adhesion assay. AB 8 cells growing in a monolayer on a 24-well culture dish were treated with 100 ng/ml rapamycin or the respective vehicle for 24 h. Medium was removed, and cells were trypsinized until all cells were suspended, which was optically controlled. Hereafter, cells were left to settle again in culture medium containing rapamycin (100 ng/ml) or vehicle for 1.5 h. Cells in suspension and adherent cells were collected separately, and cell number was measured determining LDH activity. LDH activity was assessed with a CytoTox 96w Non-Radioactive Cytotoxicity assay (Promega) following the manufacturer’s instructions. Results were depicted as percentage of adherent or suspended cells and corrected for total cell number.

Wound healing assay. Confluent differentiated AB 8, grown on six-well culture dishes, were pretreated with rapamycin (100 ng/ml) or ethanol (0.01%) for 12 h and wounded with two strokes in 90° angle with a sterile 0.4-mm 200-μl Gilson style extension length tip. Images of the same field were acquired after 0 and 10 h using an Axiosvert Microscope. The number of cells that migrated in 2 mm2 of wound area were counted.
Statistical analysis. All experiments were performed several times (3–18), and data were expressed as means ± SE. Controls and experimental conditions were compared using unpaired t-test. Statistical significance was deemed as \( P < 0.05, P < 0.01, \) or \( P < 0.001. \)

RESULTS

Influence of rapamycin on cell viability. To monitor the effect of rapamycin on podocytes, we measured the phosphorylation rate of p70S6K (p-p70S6K), a well-characterized downstream target of mTOR kinase and compared it with the total expression of p70S6K (23). Figure 1A, bottom, shows that increasing amounts of rapamycin (1 ng/ml up to 1,000 ng/ml) did not alter the expression level of p70S6K, but concentrations above 5 ng/ml rapamycin were sufficient to completely inhibit the phosphorylation of p70S6K (Fig. 1A, top). In further experiments, we used cells that were treated with low (Rapa 10) rapamycin concentration, close to the minimal inhibition dose (10 ng/ml), and a higher concentration of rapamycin (Rapa 100, 100 ng/ml).

Next, the influence of rapamycin on cell viability was characterized. The LDH assay showed that the higher amount of rapamycin treatment slightly but significantly reduced podocyte number, whereas the low concentration of rapamycin had no significant effects (Fig. 1B). Furthermore, we investigated if the reduced viability of podocytes treated with rapamycin is mainly the result of necrosis or apoptosis. The low concentration of rapamycin had no effect on the necrosis level; only the higher concentration led to a slight but significant increase of necrosis (Fig. 1C). In contrast, apoptosis of podocytes was slightly but significantly elevated only by the low rapamycin concentration (Fig. 1D). The high rapamycin concentration did not significantly change the number of apoptotic cells.

Rapamycin decreased mTOR and rictor and increased raptor expression. Next, we investigated if rapamycin changes the expression level and phosphorylation status of mTOR. Cells that were treated (24 or 120 h) with rapamycin in high and low concentration showed a significant decrease of mTOR expression level (Fig. 2A, top). The downregulation of total mTOR expression correlated with a reduced phosphorylation level of the serine residues at position 2448 (mTOR Ser2448; Fig. 2A, middle) and position 2481 (mTOR Ser2481; Fig. 2A, bottom). The longer incubation time with high rapamycin (120 h) emphasizes the trend on mTOR expression level after 24 h. Furthermore, we found a rapamycin-dependent increase of the raptor expression level that becomes statistically significant after 120 h (Fig. 2B, top). In contrast, raptor expression was dramatically decreased in cells that were treated with low and high rapamycin concentrations for 24 and 120 h (Fig. 2B, bottom, and supplemental Fig. 1 (Supplemental data for this article can be found at the American Journal of Physiology: Renal Physiology website.)).

Coimmunoprecipitation of endogenous raptor and rictor in rapamycin-treated cells. Next we tested whether rapamycin might influence the interaction of mTOR with raptor (mTORC1) and rictor (mTORC2) by performing co-IP assays with an anti-mTOR antibody coupled to beads. A Western blot against total mTOR, raptor, and rictor was used as an input (cell lysates) and empty beads as a negative control (Fig. 3, bottom and right, respectively). The coprecipitation level of raptor was only slightly decreased in rapamycin-treated podocytes. In contrast, the levels of mTORC2 were strongly reduced in co-IP assays of rapamycin-treated podocyte extracts because of a strongly decreased level of coprecipitated rictor.
Influence of rapamycin on the phosphorylation level of Akt Ser473. Akt/PKB kinase acts as an upstream regulator of mTOR. Akt in turn is activated itself by the phosphorylation of threonine-308 (p-Akt Thr308) and serine-473 (p-Akt Ser473). It is known that mTORC2 acts as an upstream regulator of Akt that promotes the phosphorylation of Akt at position Ser473. As we observed, a decreased rictor expression (Fig. 2) and markedly reduced mTORC2 complex presence in rapamycin-treated podocytes (Fig. 3), we tested if rapamycin changes the expression level of total Akt and if it alters the phosphorylation status of Akt.

The total expression of Akt and the phosphorylation of Akt Thr308 remained unchanged in rapamycin-treated podocytes (Fig. 4) and the phosphorylation level of PDK1, which regulates the Thr308 phosphorylation. An incubation for 120 h with a high rapamycin concentration led to a significant reduction of the Akt Ser473 phosphorylation (Fig. 4).

Prolonged rapamycin treatment decreased the expression of nephrin, TRPC6, and Nck. Previous studies showed that the nephrin complex is involved in regulating the Akt pathway in podocytes (17, 45). Moreover, some evidence suggests that the nephrin complex also participates in the regulation of the cytoskeleton via cytoskeletal adaptor proteins (4, 19, 26, 42). Therefore, we tested the hypothesis whether rapamycin treatment influences the expression level of proteins crucial for slit diaphragm formation or cytoskeletal adaptation.

Treatment of podocytes with rapamycin resulted in a significant downregulation of nephrin (24 and 120 h) and the Ca2+ channel TRPC6 (after 120 h, Fig. 5A). The expression of the cytoskeletal adaptors CD2AP and α-actinin 4 remained unchanged, whereas prolonged rapamycin treatment resulted in a significant reduction of Nck expression (Fig. 5B).

Rapamycin treatment of podocytes reduced cell adhesion and migration. Cell-cell contacts and the adherence of podocytes to the extracellular matrix of the GBM are crucial for podocyte function. Adhesion assays were performed (Fig. 6A) to investigate the effect of rapamycin on podocyte anchorage to the GBM and a scratch assay to analyze if rapamycin influences the migration of podocytes into the wound (Fig. 6B). Cells that were able to resettled again (adherent cells, Fig. 6A, left) or that remained in the medium (suspension cells, Fig. 6A, right) were quantified. Figure 6 shows that rapamycin treatment significantly reduced the adherence of podocytes (t-test, \( P < 0.01, n = 12 \)).

Foot processes of podocytes are highly flexible and dynamic structures and play a key role in withstanding the continuous filtration pressure. In case of damage or loss of single podocytes, the motility of podocytes is important to repair wounds.
on the capillary loop. Previous studies showed that these processes depend on rearrangements of the actin cytoskeleton (2). Wound healing assays were used to address this point. In contrast to the controls, cells that were treated with rapamycin were not capable of closing the wound within 10 h. (Fig. 6B, right). A quantitative analysis of the scratch assay revealed that rapamycin significantly reduced the migration of podocytes (Fig. 6B, left).

Rapamycin induced an increased presence of dot-like actin rich structures in podocytes. The nephrin interaction with Nck has emerged as critical for actin rearrangement and polymerization processes in podocytes (4, 19, 26, 40, 42). Therefore, it was tested whether a decreased expression level of nephrin and Nck on one and rictor (as part of TORC2) on the other hand influence the actin cytoskeleton in podocytes. As shown in Fig. 7, rapamycin caused a significantly enhanced presence of dot-like actin positive structures in podocytes (Fig. 6B, C, left). A quantitative analysis of the scratch assay revealed that rapamycin significantly reduced the migration of podocytes (Fig. 6B, left).

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least in part a result of podocyte damage. To address the question which molecular factors may be involved in rapamycin-induced podocyte injury, we investigated the effect of rapamycin on mTOR signaling and podocyte function.

Using concentrations similar to those measured in blood plasma of patients, we found that rapamycin slightly increases the apoptosis of terminally differentiated podocytes, suggesting that low rapamycin concentrations are able to induce programmed cell death via apoptosis, whereas higher amounts of rapamycin mainly cause uncontrolled cell death via necrosis. In general, these results are consistent with the findings of Laugharne et al. (22) who reported that rapamycin had no effect on podocyte viability.

We show that a prolonged rapamycin treatment led to a significant downregulation of mTOR. This decreased mTOR expression correlated with a reduced presence of the phosphoforms of mTOR Ser2448 and mTOR Ser2481. Both phosphorylations are crucial for the enzymatic activity of mTORC1 that also includes raptor. Interestingly, although mTORC1 presence was slightly reduced during rapamycin treatment, raptor itself was upregulated during rapamycin treatment. This indicates that podocytes probably increase raptor expression as an answer to the inhibitory effect of rapamycin.

The kinase PDK1 phosphorylates Akt at Thr308, whereas a "PDK2"-like factor promotes the phosphorylation at Ser473 (15). Both phosphorylations are required to activate Akt. Recently, it has been shown that mTORC2 promotes this Akt Ser473 phosphorylation at least partially, suggesting that mTORC2, in contrast to mTORC1, acts as an upstream regulator of Akt (32, 34). We observed that rapamycin treatment in podocytes dramatically reduced the expression level of rictor and that this was accompanied by a significantly reduced presence of mTORC2 (see Fig. 2B and Fig. 3).

Furthermore, we observed a significantly reduced phosphorylation of Akt at Ser473 in podocytes. In contrast, the expression level of total Akt and the phosphorylation of Akt Thr308 remained unchanged. Our findings confirm the observation of Sarbassov et al. (33) who demonstrated that prolonged rapamycin treatment (24 h) inhibits TORC2 assembly in several mammalian cell types. Therefore, we propose that mTORC2 also acts as an upstream activator of Akt in podocytes and that prolonged rapamycin treatment markedly influences the mTORC2 function.

A subunit of phosphatidylinositol (PI) 3-kinase, which activates the Akt/mTOR pathway, binds to the cytoplasmatic domain of nephrin (17). Moreover, recently, it has been shown that nephrin regulates actin rearrangements via PI 3-kinase, suggesting that the nephrin complex is physically linked to the Akt/mTOR pathway, thereby regulating the actin cytoskeleton at the slit diaphragm (35, 45). The nephrin complex in turn is tightly associated with the cytoskeleton adaptors to provide multiple binding sites for further actin-binding proteins (12, 16, 17, 36). We found that prolonged rapamycin treatment does not only change the balance between mTORC1/C2 components but also decreases the expression level of nephrin, TRPC6, and the cytoskeletal adaptor Nck (see Fig. 5B) was not changed.
Nephrin directly interacts with Nck, which is interesting, since Nck proteins are adaptors that act as key regulators of actin polymerization (4, 19, 26, 42). Dominant negative mutants of several actin-associated proteins or knockouts of these proteins in podocytes cause rearrangements of the actin cytoskeleton of podocytes that result in the destruction of the slit diaphragm structure and a loss of adherence (foot process effacement). Therefore, rapamycin-dependent reduction of actin cytoskeleton adapters and/or core components of the slit diaphragm like nephrin might be responsible for rapamycin-associated proteinuria (12–14, 21, 25). In this context, the reduced cell adhesion and cell motility induced by rapamycin as well as the formation of actin-rich dot-like structures, interpretable as centers of actin rearrangement, might contribute to podocyte injury because both cell motility and cell adhesion are strongly actin dependent processes and are crucial for podocyte function (21, 37).

Previously, Etienne-Manneville et al. (18a) showed that TORC2 controls the actin cytoskeleton, acting as an upstream regulator of Rac GTPases, suggesting that a reduced mTORC2 formation could contribute to impaired podocyte function. Therefore, we tested whether rapamycin treatment altered the endogenous activation level of Rho GTPases in rapamycin-treated podocytes (Fig. 7). Interestingly, activation levels for all three GTPases were unchanged, suggesting that the changes are minor and/or additional factors contribute to the reduced motility/adhesion and to actin rearrangements. Moreover, it is also possible that the observed changes mainly correspond to a relocalization of GTP-bound Rho GTPases and not to an increase of the GTP-bound conformation of these GTPases. In addition, there are further factors that regulate cytoskeletal rearrangements than members of the GTPases of the Rho family. One example is the integrin-linked kinase (ILK) complex.

McDonald et al. (28) recently found that rictor directly interacts with ILK. The ILK-rictor complex is able to phosphorylate Akt at position Ser\textsuperscript{473}, similar to mTORC2, suggesting that the ILK-rictor complex is at least partially able to...
phenocopy mTORC2 function. In this context, it is interesting that it has already been shown that the loss of ILK in podocytes results in severe GBM alterations and progressive glomerulosclerosis (7, 10, 39). This implicates that prolonged rapamycin treatment of podocytes might not only interfere with the formation of mTORC1 and mTORC2 but also with the assembly of a proper ILK-riktor complex, which makes the relations between mTOR complexes and the actin cytoskeleton even more complex.

Our observations provide several molecular links to explain how rapamycin is able to cause heavy proteinuria in patients. It is noteworthy that proteinuria and nephrotic syndrome are not developed by all patients treated with rapamycin. This strongly suggests that rapamycin concentration, the patients’ genetic background, preexisting damages, and/or additional factors like the time point of conversion from CNI to rapamycin or immunological and nonimmunological effects contribute significantly to the onset of rapamycin-associated proteinuria. Our results underline the complexity of the effects of rapamycin on podocytes. We identified molecular links that connect mTOR inhibition to slit membrane and cytoskeleton alterations, thereby presenting a model to explain reduction/loss of podocyte motility and adhesion and ultimately the onset of proteinuria. Our data give an impulse for further investigations of the discussed pathways and molecules affected by rapamycin. A better understanding of the molecular mechanisms triggered by rapamycin in the podocyte will help to elucidate which patients will ultimately profit from rapamycin treatment.

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Fig. 7. Rapamycin caused dot-like actin-rich structures in podocytes. Cells were treated with rapamycin (Rapa 100) or the solvent (control) and stained for actin, vinculin, zyxin, and cortactin (A-C, top, magnification ×630, details marked with white rectangles are given in bottom). The cortical ring in rapamycin-treated cells is exemplary marked with arrowheads (A, left); the rapamycin-induced actin-rich dot-like structures (arrows) were shown in higher magnifications (A-C). Merged images are given in right of A-C. D: statistical analysis showed that the rapamycin-dependent formation of dot-like structures is statistically significant (P < 0.001; n = 6). E: the expression level of vinculin, zyxin, and cortactin in treated and untreated immortalized podocytes remained unchanged. F: Rho, Rac, and Cdc42 GTPase activity assays of cell extracts from rapamycin-treated cells and solvent controls (top). Determination of ratio between GTP-loaded and the amount of total Rho, Rac, and Cdc42 GTPases (bottom).
RAPAMYCIN EFFECTS ON IMMORTALIZED PODOCYTES

The work contains parts of the MD thesis of M. Wolfgart.

GRANTS

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REFERENCES


